ESSENTIAL GUIDES TO METHOD DEVELOPMENT IN AFFINITY CHROMATOGRAPHY

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Introduction

Highly selective affinity-based separations have evolved considerably over the past two decades to improve characteristics related to target specificity, dynamic adsorptive capacity and chemical robustness of the affinity matrix. These separation matrices are used as screening devices for molecular interactions as well as for the purification of complex mixtures at the analytical, preparative and large scale. Affinity adsorptive phases can be synthesized by the attachment of structures called ligands to immiscible polymeric fluids, solvated gels and porous solids. Ligands are selected for their affinity to a soluble or colloidal target species. Advances in technology have been associated with the synthetic and biosynthetic design of affinity ligands and matrices. These advances have been made through a better understanding of phenomena associated with the transport of the target species to the ligand and the nature of molecular interactions between target and ligand. Specialized processing equipment for large and small scale applications has also improved affinity separations.

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Matrix Materials and Processing Geometries

Separations achieved by the selective capture of a target from a fluid phase by an affinity ligand confined to an adsorptive phase are most often done in aqueous systems. Therefore, the adsorptive phase for a hydrophilic target species is often a hydrophilic material. More hydrophobic targets require matrices which offer a more hydrophobic environment. In some cases, the adsorptive phase can be a separate fluid phase or soluble component having affinity ligands which will capture and concentrate the target species from complex aqueous mixtures.

Mattiasson demonstrated that heterobifunctional ligands could be used for selective isolation of proteins by affinity precipitation. Heterobifunctional ligands can bind the target and are also covalently coupled to a polymer which can be used to induce precipitation of a target/ligand complex. Alternatively, multiple but identical binding sites can be occupied on the target by homobifunctional ligands. This results in a cross-linked network between targets which can be precipitated. Polymers used for affinity precipitation applications include chitosan, alginate, polyethyleneimine, and Eudragit S-100 (a copolymer of methyl methacrylate and methacrylic acid). By manipulating such parameters as the pH or temperature, the polymer/ligand/target complex can be reversibly rendered soluble and insoluble. Lactate dehydrogenase and alcohol dehydrogenase have been precipitated using Cibracron Blue 3GA coupled with Eudragit™ S-100.

A significant advance in the field of bioseparations is the coupling of affinity ligands to aqueous two-phase systems of water-soluble polymers. Affinity partitioning is the selective extraction of target proteins from crude mixtures using affinity ligands immobilized on to either of two incompatible, water-soluble polymer phases. Mattiasson used triazine dyes coupled to immiscible polymer fluids to purify proteins by affinity partitioning. Reactive triazine dyes have been widely exploited using this separation technique. In comparison to classical chromatographic systems, affinity equilibrium is attained more rapidly in aqueous two-phase partitioning. In addition, high affinity binding capacities and protein recoveries have been achieved. Alternatively to two-phase aqueous polymer partitioning, McCreath and Chase immobilized human immunoglobulin G on to perfluorocarbon emulsions for the selective adsorption of *Staphylococcus aureus* cells containing membrane-bound protein A. These liquid emulsion droplets were comprised of a perfluorocarbon oil cross-linked with poly(vinyl alcohol).

Most of the applications for affinity-based separations are developed for chromatographic processing of aqueous systems. Thus, the ligand is immobilized on a porous bed of solids packed in a column through which liquid can be pumped. Most of the matrices are made of hydrogels composed of cellulose, agarose and dextran. A chief advantage of hydrogel matrices is the combination of an easily derivatized, hydrophilic environment that provides molecular accessibility that can be extended to 10^6 Da. Other semi-synthetic and synthetic polymeric matrices, such as copolymers of vinyldimethyl azlactone and methylene-bis-acrylamide, can also provide similar

intraparticle transport features. Coleman immobilized protein A at high density on to azlactone-functionalized polymeric matrices. These affinity matrices demonstrated high throughput capacity combined with low operating pressures. As an alternative to the use of pellicular matrices or matrices comprised of reduced bead particle diameter, Rodriguez and Liapis described the mechanism of perfusion chromatography in which intraparticle mass transfer resistance is reduced by increasing the particle permeability. Velander optimized the molecular accessibility and mechanical stability of uncross-linked cellulose adsorbents by using large diameter beads (>0.3 mm) which operate at low pressures even at high flow rates. These hydrogel matrices had a solids content of only 3% or less and were shown to provide fast intraparticle transport, apparently through a mixed mode of both diffusion and convection. Larsson demonstrated the use of superporous agarose matrices. The agarose beads contained a typical internal porous network in addition to larger pores. These larger pores constituted a significant portion of the bead, up to one-third to one-tenth of the bead particle diameter. Mass transfer characteristics were improved since the larger pores allowed a considerable fraction of the bulk chromatographic flow to penetrate and flow through the individual bead particles. Affinity matrices were prepared using these superporous agarose beads containing immobilized NAD^+ analogue for the isolation of bovine lactate dehydrogenase and protein A for the isolation of rabbit immunoglobulin G. These affinity matrices operated under much higher processing flow rates compared to conventional homogeneous bead columns. The protein A and NAD^+ analogue matrices had processing throughputs five and three times higher, respectively, in comparison to processing throughputs demonstrated by conventional affinity matrices. Thus, a significant improvement to affinity technology is the design of matrices having greater intraparticle accessibility and transport of the target species.

Advances in transport phenomena needed for target contacting with the affinity adsorbent have resulted in matrix designs having large or small particulate as well as membrane geometries. Smaller particles, having mean diameters of about 0.1 mm or less, yield a greater surface area to volume for improved target mixture contacting, but higher drops when operated in a packed bed mode. Thus, specialized affinity separations using high pressure liquid chromatography have been developed for small and large scale. However, these media require pre-clarification of the feed stream before chromatographic processing to prevent column fouling. Alternatively, small diameter particles having a higher density than water can be fluidized to achieve both filtration and affinity adsorption in a single step using expanded bed chromatography. Expanded bed adsorption chromatography has been developed to provide single stage operation for the isolation of target proteins from crude mixtures such as milk, hybridoma cell culture fluid and fermentation broth. For example, humanized lgG4 antibody was isolated from myeloma cell culture fluid by expanded bed adsorption using recombinant protein A immobilized on to Pharmacia Streamline™ media.

Planar membranes are often used for affinity separations in analytical assays but some cross-flow, stacked sheet geometries have been developed for large scale applications. Affinity membranes for large scale work can also be cast in tubular hollow fibres. One example of planar membranes was made of a gel formed from two dispersed liquid phases. The chief advantages of affinity membrane systems are the low pressure operation and fast intraparticle transport due to the short diffusion distances in thin membranes. Thus, the kinetics of ligand/target interactions can become rate limiting to affinity separations using thin membranes. Etzel showed that limitations in membrane performance can arise due to variations in porosity and thickness which can result in diffuse breakthrough loading profiles. The stacking of planar membranes can sharpen affinity breakthrough profiles at higher loading flow rates.

Affinity Ligand Selection and Design

Affinity ligands have evolved from antibodies, enzymatic substrates, co-factors, nucleic acids, coenzymes, hormones, immunoglobulin, lectins, effectors and inhibitors to a great diversity of small, low molecular weight peptides, polypeptides and other organic structures. These newer classes of ligands can be made using biosynthetic and wholly synthetic methods. Common to all selection strategies is the need to begin with a diversity of structures from which to discover candidate affinity ligands. The chance discovery of a ligand with desirable properties has been enhances through the use of phage display and synthetic combinatorial libraries which contain many orders of randomized structural permutations. The structural permutations within a library rely on the polymeric assembly of bi- or multifunctional monomeric molecules into compounds which are typically greater than 10^3 Da in molecular mass. In the case of biosynthetic libraries, the assembled structures are necessarily polypeptides and the subunits are amino acids. In the case of wholly synthetic structures, a variety of bi- and multifunctional organic

subunits that provide high selectivity and yield reactions have been sequentially assembled using soluble or solid-phase linked chemistries. For example, various perturbations of bifunctional molecules having reactive oxazoline chemistry have been used to create assembled combinatorial chemical libraries having 10^3 or more structures. In addition, some wholly synthetic libraries have used amino acids and novel branched chain peptide linkages as core structural platforms.

In 1985, Smith demonstrated the use of phage technology to display candidate polypeptide ligands from a bacterial virus particle which also contains the DNA encoding the polypeptide sequence. The polypeptide is displayed from the virus particle surface in a manner that also enables affinity interactions with a targeted species. Thus, candidate ligands can be screened by the specific adsorption of phage to target species which have been immobilized either to a microtitre assay plate or to a chromatographic matrix. Nonspecifically adsorbed material is then washed away and the specifically adsorbed phage particles are eluted. The eluted phage particles are cultured using microbiological plate-streaking techniques. Candidate phages from the first affinity screening are passed through a second affinity screening and reculturing. Phages obtained from the two affinity and culture selection processes are subjected to DNA sequencing. The DNA sequence of the displayed polypeptide is readily determined because of specific endonuclease restriction sites engineered into the phage genome. The polypeptide ligand DNA sequence then is readily used to create ligands in mass quantity through recombinant fermentation technology. About 10^8 polypeptides can be created within a single phage display library where about 10^1 to 10^2 candidate ligands typically result after a sequence of two affinity and culture screenings. Thus, the chief advantage of the biosynthetic phage display libraries is the large number of library members and facile screening due to the coupled nature of the encoding DNA and displayed polypeptide ligand of the phage particle. The chief disadvantage is the inherent limitation to structures which are polypeptides. However, as a further improvement of phage library technology, Ladner demonstrated that the use of random permutations about a core polypeptide ligand sequence can greatly enhance the affinity of initial ligand candidates. The chemical robustness of polypeptides is typically limited to moderate pH and aqueous environments.

Examples of the use of phage-display libraries in the development of affinity ligands are found throughout the recent literature. Ladner generated a series of libraries comprised of variants of the first Kunitz domain of human lipoprotein-associated coagulation inhibitor (tissue-factor pathway inhibitor-I). A typical human Kunitz domain was chosen as the parental protein since immunogenicity would be minimal due to its human origin and lack of glycosylation which would facilitate the use of phage-display technology. The library was screened against human plasmin, which is a serine protease that participates in the fibrinolytic process. This study synthesized a protease inhibitor exhibiting a high affinity and specificity for plasmin. Small ($\sim$58 amino acid residues), stable Kunitz domains, which lack glycosylation, and containing nearly human sequences were selected and determined to have high affinity and specificity towards plasmin. The same phage-display library and screening methodology has been used to select high affinity and high specificity ligands for human plasma kallikrein and human thrombin. Markland demonstrated that certain constraints may be imposed upon primary and tertiary structures to increase specificity relative to more simple linear peptide ligands directed towards the same target. For example, a phage-display library was constructed that displayed an 18 amino acid residue peptide containing two fixed cysteine residues to allow disulfide bond formation. In addition, several variable residue positions between and adjacent to the two cysteine residues were included in the peptide sequence. This library was screened against streptavidin and an anti-β-endorphin monoclonal antibody. The screening yielded phage displaying disulfide-constrained microproteins. The selected phage clones required a disulfide bond for the high affinity binding to both target proteins. Other core peptide motifs have resulted in phage-display libraries displaying protease inhibitory domains derived from wild-type bovine pancreatic trypsin inhibitor. In one case, Ladner selected a ligand which was an inhibitor of human neutrophil elastase that had a 3.6 million-fold higher affinity than that for the parental protein and was reported to exceed the highest affinity cited for any human neutrophil elastase inhibitor by 50-fold.

Wholly synthetic combinatorial libraries typically use a robotic assembly of structures. The robotic equipment used to generate orders of structural permutations essentially consists of a miniaturized chemical factory. These multifunction automated work stations perform sequences of sample mixing, thermostatic reactions, volume reduction and purification of the reaction masses. The identity of individual reaction masses is catalogued. Identification of the newly created chemical structures is deduced from automated combinations of liquid chromatography and mass spectrometry. Libraries having 10^3 to 10^4 distinct members have been synthesized and

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characterized in this way. The screening of wholly synthetic libraries is laborious due to the need to putatively identify ligand/target complexes and subsequently to test the ligand structure in an immobilized state. In summary, a chief disadvantage of these libraries is the many order fewer library members that can be generated using the current robotic methods. Another disadvantage is cumbersome information acquisition and management associated with both the characterization of the ligand structure and target affinity. Unlike phage display, the primary molecular structure is not encoded and coupled to the wholly synthetic ligand as enabled by DNA of the phage particle. However, the diversity of chemically robust structures that can be produced from wholly synthetic molecules is an important advantage. In addition, rational design models can greatly decrease the number of permutations necessary from the initial discovery of ligands having lower affinity to the design of high affinity ligand candidates based upon initially discovered, ligand structural motifs. If combined with rapid and sophisticated assay screening methods, combinatorial methodologies provide an efficient process for the development of novel drug leads and affinity ligands. Future improvements in the efficiency of screening combinatorial ligands will likely be made using automated chemical sensor technology.

Affinity peptide ligands can be made using solid-phase chemistry. Houghten showed that the solid-phase-linked chemistries have advantages of separation/wash cycling between reaction sequences as well as being able to provide information about the base structure of the candidate ligands. Independent studies by Pingali and Fassina have demonstrated the use of affinity peptide ligands generated from solid-phase peptide synthesis. Pingali produced an affinity peptide that bound human fibrinogen and a peptide of chromochrome c containing haem that captured human serum albumin (HSA). The anti-fibrinogen tetrapeptide was made using standard FMOC (*N*-fluorenylmethoxycarbonyl) chemistry. The affinity matrix made from this peptide was highly specific as purified fibrinogen was obtained directly from crude human plasma. Frontal analysis determined that the dynamic binding capacity of the antifibrinogen column was 10.2 mg fibrinogen per millilitre of gel. Similarly, by frontal analysis, an HSA-affinity peptide column was found to have a dynamic binding capacity of 19 mg HSA per millilitre of gel. Fassina used a core motif consisting of a small branched-chain peptide to discover a protein A mimetic affinity ligand. This peptide recognized the Fc fragment of immunoglobulin G from rabbit, goat, sheep and mouse and provided a one-step isolation method for highly purified immunoglobulin G directly from crude sera. Frontal analysis determined that the dynamic binding capacity was 2 mg rabbit immunoglobulin per millilitre of gel. The peptide also exhibited stability towards sanitization reagents such as 0.1 mol L^{-1} sodium hydroxide and ethanol. Both of the above-mentioned studies show that peptide-based affinity columns derived from standard solid-phase peptide synthesis can provide a viable alternative to the use of immunoaffinity chromatography.

Molecular modelling is used to deduce likely interactions between the target and affinity ligand, and is frequently used as a basis for further rational design of both biosynthetic and wholly synthetic combinatorial libraries. Once certain nominal affinity motifs have been identified from initial ligand screening, rational design has been used to enhance affinity by creating permutations which build upon structural motifs having lower affinity for the target species. In addition, recent studies have been conducted which attempt to develop 'peptidomimetic' structural compounds, such as aminimides, which have core structures similar to that of peptides, but with improved characteristics. Aminimides are much more chemically stable, have enhanced solubility characteristics and are resistant to enzymatic degradation. Ringe formulated a method for structure-based design involving aminimides in which complete binding surfaces of the targets are mapped. Combinatorial chemistry is then used to identify and improve structural specifications in lead candidates targeted at the corresponding binding sites. Ringe developed a peptidomimetic aminide inhibitor or porcine pancreatic elastase based upon crystal structures of an aminimide analogue.

Affinity dyes are another class of wholly synthetic affinity ligands which are not derived from combinatorial libraries. Reactive synthetic textile dyes are resistant to chemical and biological degradation and can bind selectively and reversibly to a wide range of enzymes and proteins. These ligands evolved from ligands which were first classified as enzyme co-factor mimetics. Many of these co-factor mimetics were members of reactive dyes used for staining textiles and paper. Since the mid-1950s, these reactive textile dyes have been used in the affinity purification of proteins. The use of commercial textile dyes as ligands in the large scale processing of diagnostic, therapeutic and genetically engineered proteins is documented throughout the literature. Since that time, many variations of original dye ligand structures, such as reactive Cibacron Blue, have been synthesized. These new structure–function motifs are no longer considered a simple co-factor mimetic relationship as was recognized between nicotinamide

and Cibacron Blue. Cibracron Blue F3G-A, the most thoroughly studied dye ligand, binds with a variety of proteins such as adenine coenzyme-dependent oxidoreductases, phosophokinases, hydrolases, transferases, nucleases, polymerases, synthetases, lyases, decarboxylases, in addition to glycolytic enzymes and plasma proteins. Lowe has shown that the triazine dye structure binds to clefts of proteins which have no known co-factor binding domains, but have much more subtle, yet specific interactions with triazine dye derivatives. Molecular modelling has also improved the rational affinity design of triazine derivatives through the addition of various chemical moieties to the core triazine structure. Lowe demonstrated the use of computer-aided molecular modelling and design in the development of structurally modified biomimetic dyes based on Cibracron Blue F3G-A and Procion Blue MX-R. A dye ligand was engineered specifically for the capture of horse liver alcohol dehydrogenase.

There are several advantages to the purification of pharmaceutical products using synthetic affinity ligands. A major advantage is that they are not derived from biological sources. Therefore they impart less of a product contamination risk. Hence, regulatory issues concerning the presence of unknown contamination and infectious agents in the final product are circumvented. Other major advantages of these ligands include: lower manufacturing cost; they can be readily immobilized under a wide variety of coupling chemistries to an extensive range of commercially available supports; they can be modified to enhance specificity or stability; and they are more stable and less susceptible towards denaturation. In addition, ligands derived from combinatorial libraries may contain a diversity of novel molecular structures such as organically derived or non-natural amino acid residues such as L-amino acids (D-optical isomers). Well-characterized affinity ligands may ultimately dictate decreased final product costs associated with less expensive process costs (i.e. more robust affinity matrices and less expensive ligands) and higher throughput due to a decrease in number of required chromatographic steps (i.e. decreased buffer usage). Affinity separation of pathogens may be better, enabled by the increased accessibility of small ligands to subtle, yet conserved domains of viruses.

Installation of Affinity Ligands

The covalent installation of the affinity ligand into a chromatographic or membrane-based matrix can profoundly affect the ligand/target binding efficiency. Matrix coupling chemistries usually covalently attach ligands through highly reactive groups such as α-amino groups. Common activation methods for polysaccharide matrices are cyanogen bromide, divinylsulfone, epoxy, organic sulfonyl chlorides, carbonyl diimidazole, and *N*-hydroxysuccinimide. Polyacrylamide matrices are commonly activated by using glutaraldehyde or hydrazine. Isothiocyanate and γ-glycidoxypropylsilane activation methods are commonly used for silica-based matrices.

Ligands which contain amino acids can be coupled through the ε-amino group of lysine, carboxyl groups of aspartate and glutamate, and the phenolic group of tyrosine. Chemistries which randomly couple these residues also result in random orientation and spacing of the immobilized ligand. Some of the most thorough studies of ligand coupling chemistries have been done with proteins, especially antibodies. For example, Velander demonstrated that antibodies used as affinity ligands exhibit best performance characteristics when the binding conformation of the antibody is protected during covalent coupling to the adsorbent phase. A conformationally related effect is the orientation of the immobilized ligand. The use of orientated immobilization methods for antibodies is also applicable to synthetic and biosynthetic affinity ligands that have asymmetric structure due to the presence of both target binding and nonbinding domains. The nonbinding domain is best used for covalent coupling. Coupling of ligands to the support matrix through reactive moieties present within the binding domain can be detrimental from both orientation and non-native conformational effects acting upon the ligand. However, masking or shielding of the binding domain prior to immobilization can be employed to circumvent these effects. Velander used synthetic antigens consisting of water-soluble adducts of poly(2-methyloxazoline) polymers and a synthetic peptide epitope for the masking of monoclonal antibody during immobilization. The mask was then removed from the immobilized antibody. A loss of as much as 50% of the theoretical binding capacity of an immobilized antibody was attributed to orientation effects based upon anti-Fc and anti-Fab probing of immunosorbents made using masked and unmasked antibodies.

Activated matrices which preferentially couple through specific functional groups on ligands can aid in the site-directed, orientated immobilization of affinity ligands. For example, Domen developed several activated matrices in which each couple antibodies through different functional groups. Iodoacetyl groups on SulfoLink™ gel are designed to couple through sulfhydryl groups found predominantly in the hinge region of antibodies. CarboLink™ activated gels couple through aldehyde groups. The aldehyde groups on antibodies can be formed by the

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oxidation of carbohydrate found primarily in the Fc region of antibodies. The site-directed method of coupling for a bivalent antibody through the oxidized carbohydrate groups using hydrazide chemistry resulted in the theoretical maximum of two antigen molecules for every antibody immobilized. However, Velander and Orthner found some antibodies immobilized on to matrices using hydrazide chemistry, which couple through the carbohydrate groups, produced no significant differences in immunosorbent antigen binding efficiency in comparison to immunosorbents prepared using random coupling chemistries such as cyanogen bromide. These antibodies were found to have carbohydrate in the binding domain.

The covalent attachment of the ligand through spacer arms becomes necessary when the nonbinding domain of the ligand does not offer sufficient molecular spacing between the molecular structure of the adsorbent and the binding domain to enable unencumbered binding of the target. The use of spacer arms is well documented throughout the literature. For example, Cuatrecasas demonstrated that extension of the ligand from the matrix through a hydrocarbon spacer arm can considerably increase ligand/target binding efficiency. Cuatrecasas prepared specific staphylococcal nuclease affinity matrices by immobilizing the competitive inhibitor, pdTp-aminophenyl, using spacer arms of varying length on to agarose and polyacrylamide matrices. Affinity matrices prepared with the ligand attached directly to cyanogen-bromide-activated Sepharose™ yielded a binding capacity of 2 mg nuclease per millilitre of gel whereas affinity matrices, prepared using a ligand attached to the support through a 3,3′-diaminodipropylamine spacer arm, yielded a binding capacity of 10 mg nuclease per millilitre of gel. In addition to ligand proximity to the support surface, the proximal spatial positioning of adjacent ligands within the support influences binding efficiency as well.

A high local ligand density can also encumber target binding between proximally immobilized ligands. Velander also demonstrated that antibodies immobilized with a low, local density gave as much as 50% of theoretical capacity based on a 2:1 target/antibody, molar stoichiometry. Thus, the majority of activity loss associated with the installation of antibodies into matrices can be attributed to local density and orientation effects. As mentioned above, intramatrix transport phenomena can also affect affinity sorbent performance, particularly for large target molecules. Thus, ligands are best installed into domains with rapid target accessibility. However, orientation, spacer arm and local spatial ligand density effects must be evaluated at any location within the adsorptive matrix as part of the affinity sorbent optimization process.

Future Developments

A diversity of new wholly synthetic affinity ligands will be synthesized by microfluidic devices which will essentially be 'micro-chemical factories on a chip'. Engineering affinity matrices for optimum performance will also include evaluating different immobilization environments to enhance ligand/target interactions. Since many different target binding environments may need to be screened for a given ligand, miniaturized matrices installed on sensors will likely replace the inefficient batch and chromatographic analysis of optimal immobilization environments. Older pharmaceutical processes, such as blood plasma fractionation, will eventually be supplanted by affinity separation processes.

See also: **II / Affinity Separation:** Affinity Membranes; Affinity Partitioning in Aqueous Two-Phase Systems; Convalent Chromatography; Dye Ligands; Hydrophobic Interaction Chromatography; Immobilised Boronates and Lectins; Immobilised Metal Ion Chromatography; Immunoaffinity Chromatography; Imprint Polymers; Rational Design, Synthesis and Evaluation: Affinity Ligands; Theory and Development of Affinity Chromatography; **Chromatography:** Protein Separation. **Appendix: 1/Essential Guides for Isolation / Purification of Enzymes and Proteins. Essential Guides for Isolation/Purification of Immunoglobulins.**

Further Reading

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ESSENTIAL GUIDES TO METHOD DEVELOPMENT IN CAPILLARY ELECTROPHORESIS

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Introduction

Capillary electrophoresis encompasses a number of related separation approaches, some of which are adapted to the requirements of specific applications (**Figure 1**). They share in common the use of electrolyte solutions as mobile phase, the use of capillary tubes as the separation column, and the use of an electric field to induce sample and mobile phase transport. This allows a similar instrument platform to service all capillary electrophoretic separation techniques with only minor modifications for specific applications. Detection is usually by UV-visible absorption through the fused silica capillary wall, or occasionally by fluorescence, electrochemical or mass spectrometric detection. Contemporary instruments are also highly automated for ease of use and improved control of critical experimental variables.

Classification of capillary electrophoretic techniques according to their usual applications is given in **Table 1**. These techniques can be considered as general, sample-type specific, in an early development phase, or of minor importance. Such a broad range of descriptive terms requires further elaboration to indicate how we propose to treat these techniques in this article. Capillary zone electrophoresis (CZE), or simply capillary electrophoresis, and micellar electrokinetic chromatography (MEKC) are widely used and complementary techniques for the separation of ionic and neutral molecules. They are the most important and general in terms of the number of applications and frequency of use. Capillary electrochromatography (CEC) is a relatively new and promising technique with a range of applications similar to liquid chromatography. Since electro-driven flow has been shown to provide both theoretical and practical advantages over pneumatic-driven flow, it has the potential to become a major separation technique. At present, too little is known about the technique to provide a definitive guide to method development, especially as in the future it is likely that new column materials will be developed specifically for capillary electrochromatography with properties different to those currently used. Capillary gel electrophoresis (CGE) is an important technique for the separation of biopolymers but is little used outside of laboratories that perform this type of analysis. Capillary isoelectric focusing (CIEF) is a specialized technique within the field of macromolecule zwitterion separations,